

Hyperoxia induces the apoptosis of alveolar epithelial cells and changes of pulmonary surfactant proteins

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Abstract. – **OBJECTIVE:** To explore the changes of surfactant protein C, D (SP-C, SP-D) and apoptosis of alveolar epithelial cells (AEC) in the lung injury of neonatal rats induced by hyperoxia.

MATERIALS AND METHODS: We divided neonatal rats within 24 hours into two groups randomly: the air group (n = 50) and the hyperoxia group (n = 50). Rats in the air group and hyperoxia group were bred conventionally and in tanks with normal pressure and 90% concentration of oxygen, respectively. On the 1st, 3rd, 7th, 10th, and 14th day after exposure, lung tissue of 8 rats in each group was collected and stained with hematoxylin and eosin (HE). We observed and recorded pathologic changes of lung tissue and detected apoptosis rate of alveolar epithelial cells by TUNEL (TdT-mediated dUTP nick end labeling). We detected the content of SP-C, SP-D in bronchoalveolar lavage fluid (BALF) by ELISA (enzyme-linked immunosorbent assay).

RESULTS: In the air group, the alveolar formed gradually with equable size and regular shape during growth. While the amount of alveolar decreased gradually and we observed small vessels dilation and increasing hemorrhage as well as increasing interstitial cells and swollen lung tissue in the hyperoxia group. In the air group, the content of SP-C in the BALF reduced during growth. However, the content of SP-C in the hyperoxia group was lower than that in the air group on the first day, higher on the third day, reached the peak on the seventh day and began to decrease on the tenth day and more obviously on the fourteenth day. The level of SP-D in the air group declined gradually with growing. On the first day, the content of SP-D in the hyperoxia group was similar to that in the air group. It began to increase on the third day, reached its peak on the seventh day, and began to decrease on the tenth day, more obviously on the fourteenth day.

CONCLUSIONS: Long-term exposure to hyperoxia inhibits the development of alveolar. The apoptosis of alveolar epithelial cells in-

creased and the content of SP-C, SP-D in the lung tissue first increased and then decreased with the increase of exposure time.

Key Words:

Hyperoxia, Alveolar epithelium cells, Surfactant protein, Rats.

Introduction

With the improvement of treatment of neonatal clinical illness, the survival rate of premature infants increases as well as the occurrence of bronchopulmonary dysplasia (BPD)^{1,2}. To increase the blood oxygen partial pressure effectively and improve blood supply to heart, brain, kidneys and other organs, hyperoxia therapy are often used when rescuing lives. But long-term inhalation of high concentration oxygen causes lung injury even distant organs such as retina, brain, and intestines³⁻⁵ which will infect life quality of newborns seriously. BPD, characterized histopathologically by alveolar developmental disorder and pulmonary fibrosis, is one of the most common late complications in premature babies⁶. The apoptosis of alveolar epithelial cells (AEC) is an important histologic feature of lung injury induced by hyperoxia⁷⁻⁹. The objective of the current study was to track the development of lung injury induced by hyperoxia by detecting the dynamic changes of SP-C, SP-D in neonatal rats.

Materials and Methods

Making Animal Models and Grouping

Healthy, clean neonatal male or female Wistar rats within 24 hours were provided by the Experimental Animal Center of University. The animal

use in this study has been reviewed and approved by the Ethics Committee. According to the reported natural elimination rate of hyperoxia, we increased samples by 20% (air group=50; hyperoxia group = 50) with weight ranging from 9.0 to 11.0 g. Neonatal rats in the two groups were all reared together with the mother. The newborn rats of the air group were fed in the atmospheric air environment while neonatal rats in the hyperoxia group were fed in glass oxygen chamber (a self-made glass container with 3 holes and a size of 60 cm *40 cm *30 cm) with oxygen input continually. We provided oxygen for the hyperoxia group with a 40-liter oxygen tank 23 hours a day. The oxygen tank was changed daily when we opened the chamber. The oxygen concentration maintained at about 90%. We monitored oxygen concentration at least 3 times a day by digital oxygen measuring instrument. Soda lime was used to absorb CO₂ to maintain its concentration at < 0.5%. The temperature and humidity were kept at 26°C-27°C and 60%-75%, respectively. We opened the chamber for 1 hour every day in order to observe the status of animals, add water, food and change bedding. Mother rats were exchanged between the two groups each day to avoid the decline in nursing ability or death caused by oxygen toxicity.

Specimen Collection

On the 1st, 3rd, 7th, 10th, and 14th day after exposure, 8 rats in each group were chosen randomly. After intraperitoneal injection of 5% chloral hydrate, the abdominal cavity was opened immediately, and lung tissue was separated, taken out and dried under sterile conditions. We chose the left lower lobe and fixed with 4% poly formaldehyde. Routinely, two pieces of paraffin sections were made: one for HE staining to observe the morphology of lung tissue; one for detection apoptosis of AEC. We injected 1ml saline from the right main bronchus and repeated lavage 3 times. The collected BALF (almost 80%) was centrifuged at 2000 r/min with a centrifugal radius of 8 cm for 10 min. We collected 2 ml supernatant, preserved at -70°C for detection.

Lung Tissue Morphology Observation

Paraffin sections went through dewaxing, hydration, HE staining and neutral gum sealing. The morphological changes of lung tissue were observed by a light microscope ($\times 40$).

Apoptosis Detection of AECs

Apoptosis of AECs was detected by TUNEL (the kit was purchased from Shanghai Roche Corporation of China). The prepared sections

went through dewaxing, hydration, protease treatment, closing and fixing and reacted with sample DNA under terminal deoxynucleotidyl transferase (TDT). Then the above sections were colored with diaminobenzidine, stained with hematoxylin and sealed. The cells stained with brown nucleus were defined as apoptotic cells. We selected 5 representative high-power fields ($\times 400$) on each section to calculate apoptosis index. Apoptosis index = (the number of apoptotic cells /the total number of cells) $\times 100\%$.

SP-C, SP-D Detection

We warmed the supernatant of BALF preserved at -70°C at room temperature for 1-2 hours. The content of SP-C, SP-D in BALF was detected by ELISA which was carried out according to the kit instructions strictly.

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean \pm standard deviation. Comparison between groups was done using One-way ANOVA test followed by Post-Hoc test (Least Significant Difference). *p*-values < 0.05 were considered statistically significant.

Results

General State of Newborn Rats

The general state of rats in the air group was good with normal activities and regular suckling. In the hyperoxia group, the activities and suckling were basically normal on the first day. From the third day, the general state began to go bad with decreased activities and suckling. Death began to occur from the seventh day. 2, 1, and 2 rats died on the seventh, tenth and fourteenth day, respectively. The general state went worse with fewer activities and suckling on the fourteenth day.

Changes of Lung Tissue Morphology

On the first day, small and light initial alveolar began to occur in the air group with not regular shape and thick alveolar septum which was similar to that in the hyperoxia group. On the third day, the terminal air chamber decreased with more alveolar and thinner septum in the air group. While in the hyperoxia group, some small vessels dilation, petechial hemorrhages, and increasing interstitial cells were observed. The major exuded

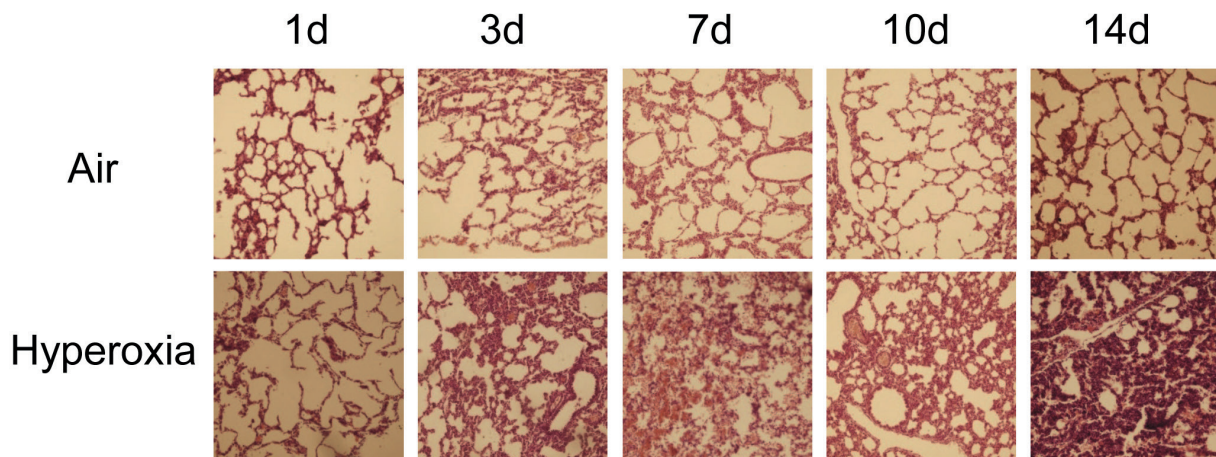


Figure 1. Pathological changes of lung tissue observed by HE staining ($\times 40$).

cells were neutrophils. Lung tissue edema was observed in the hyperoxia group. On the seventh day, alveolar in the air group developed regularly with equable size. While in the hyperoxia group, hemorrhage and edema were found in the alveolar as well as more neutrophils, increased interstitial cells, less alveolar and thicker septum. On the tenth and fourteenth day, we observed more alveolar with increased septum and equable size in the air group. However, in the hyperoxia group, the alveolar septum became thicker with multifocal consolidation, dilated alveolar cavity and reduced alveolar. Also, more inflammatory exudation and a little collagen deposition in pulmonary interstitium were found (Figure 1).

Apoptosis of Lung Tissue Cells

Statistical difference in apoptosis index was not found in the air group from the first day to the fourteenth day ($p > 0.05$). In the hyperoxia group, the apoptosis index increased gradually from the first day, peaked on the tenth day and decreased a little on the fourteenth day. The difference was statistically significant at different time-points ($p < 0.001$). There was no significant difference in apoptosis index between these two groups on the first day ($p > 0.05$). But the index in the hyperoxia group was higher than that in the air group on the third, seventh, tenth and fourteenth day statistically ($p < 0.01$) (Figure 2).

Expression of SP-C, SP-D in BALF

Statistical difference in level of SP-C, SP-D in BALF was not found in the air group from the first day to the fourteenth day ($p > 0.05$). In the hyperoxia group, the level of SP-C, SP-D

increased gradually from the first day, peaked on the seventh day and then began to decrease gradually. The difference was statistically significant at different time-points ($p < 0.05$). The level of SP-C in the hyperoxia group was lower than that in the air group on the first day statistically ($p < 0.05$). From the third day, it began to increase gradually and then declined on the fourteenth day with statistical difference ($p < 0.05$) (Figure 3). For SP-D, its content was similar to that in the air group ($p > 0.05$) on the first day. It were higher than that in the air group from day 3 to day 10 and declined on day 14 which was lower than that in the air group ($p < 0.05$) (Figure 4).

Discussion

Long-term or improper inhalation of oxygen causes cytokines abnormally increasing, imbal-

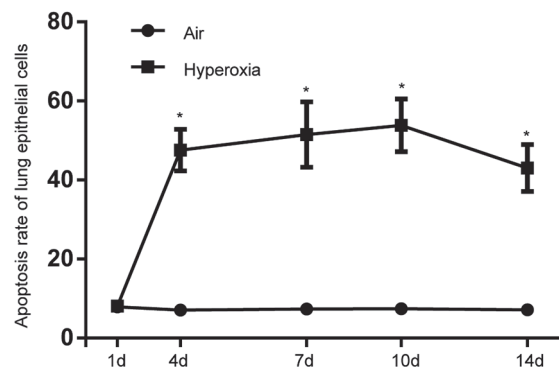


Figure 2. Apoptosis of lung tissue cells. *, $p < 0.05$ compared with Hyperoxia group.

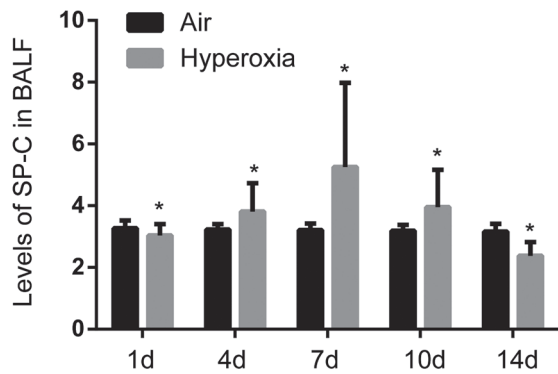


Figure 3. The expression of SP-C in BALF. *, $p < 0.05$ compared with Hyperoxia group. (SP-C: pulmonary surfactant protein-C, BALF: broncho alveolar lavage fluid).

ance of oxidation-antioxidation defense mechanism and oxidative stress increasing. All these disorders formed a regulatory network which prompts the damage of the structures and functions of AEC and alveolar endothelial cells through a series of positive and negative feedback mechanisms. As a result, lung injury and many kinds of respiratory diseases such as lung edema, BPD, and acute respiratory distress disease occur¹⁰⁻¹². The first 14 days after birth are the key period of the lung development for rats. Hyperoxia exposure during this period will affect the lung development even functions seriously. Our study indicated that the degree of lung injury increased gradually with the duration of hyperoxia exposure manifested as congestion, edema, exudation even necrosis. That was to say the longer the rats exposed to hyperoxia, the more seriously the lung

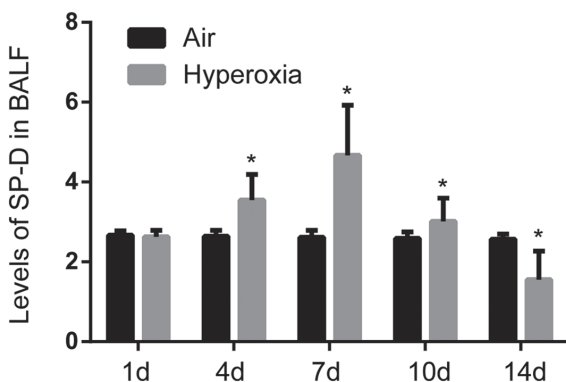


Figure 4. The expression of SP-C in BALF. *, $p < 0.05$ compared with Hyperoxia group. (SP-C: pulmonary surfactant protein-C, BALF: broncho alveolar lavage fluid).

was damaged. Lung injury induced by hyperoxia is a complex process of diffuse alveolar inflammation and repair after injury with early feature of inflammatory edema^{9,13} and afterwards pulmonary interstitial hyperplasia and finally fibrosis. In the current study, apoptosis rate in hyperoxia group was higher obviously than that in the air group and increased constantly with duration of hyperoxia exposure. Moderate apoptosis is beneficial to eliminate abnormal hyperplasia of parenchymal cells, inflammatory cells, and some exudation to recover to normal. However, abnormal apoptosis will deteriorate lung injury and cause maldevelopment of alveolar. It was reported that apoptosis in hyperoxia-induced lung injury damaged blood-air barrier and induced stroma edema, activation of fibroblasts in the repair process which lead to pulmonary fibrosis^{13,14}.

Lung surfactant proteins can be classified as SP-A, SP-B, SP-C, and SP-D which maintain the stability of pulmonary surfactant and surface tension, thereby improving the compliance, avoiding collapse of the lung^{15,16}. They are also related with pulmonary immunity. The change of SP reflects the level of PS to some extent. SP can regulate the secretion and elimination of PS to maintain the stability of the alveolar environment. Synthesized by AECII and Clara cells, SP-D, as an effective immunomodulator, participates in cellular immunity and plays a role in chemotaxis for immune cells such as mononuclear macrophages and neutrophils and opsonizing particles for phagocytosis. It can also regulate pro-inflammatory factors to protect lung injury induced by hyperoxia. SP-D exerts its effects by specifically binding and inhibiting epithelial cell surface receptors. At the same time, it is involved in the regulation of lung surface proteins which maintains normal respiratory function. Currently, few studies reported the changes of SP-C after acute lung injury. The main function of SP-C is to compose PS together with phospholipids. PS adheres to the surface of alveolar cavity and forms gas-liquid interface to decrease lung surface tension¹⁶.

SP-C can also stabilize the surface activity of surfactant membrane in respiratory motion by increasing the uptake of AECII for phospholipids. After lung injury induced by hyperoxia, the damage of AECII was significant with reduced lamellar bodies and disorder of synthesis and secretion of PS. In the process of hyperoxia-induced lung injury, the apoptosis of AECII may be a response of stress to eliminate injured cells that cannot recover to normal, thereby maintaining the stability of inner environment and limiting inflammatory response.

In this study, the longer the rats exposed to hyperoxia, the more the level of SP-C, SP-D in BALF declined which pointed out that the level of SP-C, SP-D in the BALF may be an indicator of hyperoxia-induced lung injury even the injury degree to some extent. It is reported that AECII in adult rats is relatively static and hyperoxia only causes inflammatory exudation and chronic fibrosis of AEC. But for newborn rats, especially premature rats whose lungs have not developed completely, hyperoxia could inhibit the proliferation and differentiation of AECII and the development of septum and alveolar which lead to BPD^{17,18}. Our study showed that the level of SPC in the air group did not change significantly with growing. Nevertheless, the level of SP-C in the hyperoxia group was lower than that in the air group on day 1 which indicated that AECII be injured on the first day. It is mainly due to reactive oxygen species cause the accumulation of inflammatory cells and AECII damage^{19,20}. On the third day, high expression of SP-C in the hyperoxia group was found to fight lung injury induced by hyperoxia. High level of SP-C continued to complete the defense and recovery of body on day 7. The signal transducers and activator of transcription 3 (STAT3) signaling pathway were activated by hyperoxia which promoted the synthesis and secretion of SP and protected lung tissues. On the tenth day, the level of SP-C began to decline and especially on the fourteenth day which indicated that the damage of lung tissue increased gradually with the prolongation of hyperoxia to lose compensation of self-recovery. Meanwhile, the level of SP-D in the hyperoxia group was similar to that in the air group on the first day which may be related to low-level itself. On day 3, SP-D was expressed to compensate. It peaked on the seventh day and began to decrease on the tenth day which was still higher than that in the air group. It fell to the lowest point on day 14, lower than that in the air group. The increased expression of SP-C, SP-D was caused by feedback to maintain the function of PS, fight against inflammation and then stabilize the alveolar structure and alleviate lung injury.

Conclusions

The replacement therapy of SP has developed gradually clinically. The further study of SP-D and SP-C can provide important theoretical

basis and new ideas for effective intervention and treatment of pulmonary diseases. Apoptosis participates in the whole pathological and physiological process of lung injury with a complex mechanism. To study and make clear the mechanism of apoptosis in hyperoxia-induced lung injury is of importance for searching new target and ideas for the treatment of lung injury induced by hyperoxia.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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